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ULTRASTRUCTURAL AND FUNCTIONAL EFFECTS OF LIPOPOLYSACCHARIDE AND INTERLEUKIN-2 ON HUMAN NK CELLS

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Abstract

Bacterial endotoxin (lipopolysaccharide, LPS) and interleukin-2 (IL-2) are known to stimulate NK cell mediated cytotoxicity against tumor cells. In the present report we sought to correlate the stimulatory effect of LPS and IL-2 on NK cell activity with ultrastructural changes which occurred as a result of such stimulation. Peripheral blood mononuclear cells (PBMC) were purified from healthy donors by a Ficoll-Hypaque density gradient technique. Leu-11a$^+$ NK cells were isolated by flow microfluorometry using a monoclonal FITC conjugated anti-Leu-11a antibody and a FACS II cell sorter. The PBMC were incubated, respectively, with E. coli LPS or recombinant IL-2 (IL-2) for various time periods. Sorted Leu-11a$^+$ NK cells were incubated with LPS for 24 hours. The NK cytotoxicity in the PBMC and sorted Leu-11a$^+$ cells was assessed by a ^{51}Cr release technique using K562 tumor cells as targets. Leu-11a$^+$ NK cells were identified by immunoelectron microscopy using anti-Leu-11a antibody and labeling with horseradish peroxidase or colloidal gold. Results showed that both LPS and IL-2 significantly enhanced the cytotoxic activity of PBMC. The cytotoxicity of sorted Leu-11a$^+$ cells was augmented by LPS. Recombinant IL-2 induced a significant increase in the number of dense granules, hypertrophy of Golgi apparatus and rough endoplasmic reticulum, and mitosis of Leu-7$^+$ cells and Leu-11a$^+$ cells 4 or 7 days after stimulation. These data indicate that: (1) the effect of LPS on the enhancement of NK cytotoxicity in PBMC may be a direct and/or indirect process involving production of lymphokines; (2) LPS has a direct effect on sorted Leu-11a$^+$ cells; (3) IL-2 stimulates mitosis of Leu-7$^+$ cells and Leu-11a$^+$ cells; and (4) the LPS or IL-2 induced ultrastructural changes in Leu-11a$^+$ cells are consistent with the enhanced NK cytotoxicity.

Key words: Lipopolysaccharide, interleukin-2, natural killer cells, immunoelectron microscopy, cytotoxicity, ultrastructure, cytochemistry

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Introduction

Natural killer (NK) cells are defined as a population of lymphoid cells that mediate spontaneous cytotoxicity against neoplasms, virus-infected cells, normal cells in vivo without deliberate sensitization (Herberman and Holden, 1978; Trinchieri and Perussia, 1984). NK cells are also capable of providing protection from bacterial (Lopez, 1980), and parasite (Eugui and Allison, 1982) infections. Recent evidence indicates that NK cells are also involved in regulation of growth and function of hemopoietic and lymphoid cells (Mangan et al., 1984; Hansson et al., 1982; Arai et al., 1983; Pistoia et al., 1983). In rodents (Reynolds et al., 1981; Kumagai et al., 1982; Eremin et al., 1980), felines (Herberman et al., 1980), canines (Krakowka, 1983), and human and non-human primates (Savary and Lotzova, 1986), the NK cytotoxicity is associated with a population of large granular lymphocytes (LGL) which are characterized by Fc receptors for IgG and abundant cytoplasm containing numerous cytoplasmic granules (Timonen et al., 1979; Saksela et al., 1979; Abo and Balch, 1981; Herberman et al., 1979). The ultrastructural and cytochemical studies of LGL indicate that the cytoplasmic granules contain lysosomal enzymes and are involved in cytolysis of target cells (Roder et al., 1980, 1982; Huhn et al., 1982; Grossi et al., 1982; Babcock and Phillips, 1983; Nocera et al., 1983; Zucker-Franklin et al., 1983; Kang et al., 1987a; Neighbour et al., 1982). In humans, functionally only about 80% of LGL are active NK cells (Timonen et al., 1982a), which clearly indicates that LGL, in fact, represent a heterogeneous population of cells with various phenotypes and functional capabilities.

Various surface antigens and characteristics in human LGL have been observed (Allavena and Ortaldo, 1986). Among these surface markers or antigens, Leu-7 (HNK-1), Leu-11 (NKF-15), and Leu-19 (NKH-1) are the most common and important markers, and have been broadly used to identify human NK cells in the peripheral blood, tissue fluids, and tissues (Cerf-Bensussan et al., 1983; Si and Whiteside, 1983). The recent development of the commercially available monoclonal antibodies against Leu-7, Leu-11, and Leu-19 antigens permits extensive studies on the morphological and functional properties of human NK cells. Using two-color flow cytometry, investigators have shown that human NK cells express different combinations of Leu-7 and Leu-11 or Leu-19 antigen (Lanier et al., 1983, 1986). NK cells with Leu-7$^-$/Leu-11a$^+$ or Leu-7$^-$/Leu-19$^+$ phenotype are the most potent effectors in human

peripheral blood, whereas cells expressing Leu-7$^+$/Leu-11$^-$ or Leu-7$^+$/Leu-19$^-$ are the least effective in NK activity (Lanier et al., 1983, 1984; Phillips and Babcock, 1983; Gebel et al., 1987). Our previous immunoelectron microscopic studies of human NK cells using an immunogold and immunoperoxidase double-labeling technique indicate that human peripheral blood lymphocytes (PBL) contain 5% Leu-7$^+$/Leu-11a$^-$, 15% Leu-7$^+$/Leu-11a$^+$, and 9% Leu-7$^-$/Leu-11a$^+$ NK cells (Kang et al., 1987a) and also show that effector cells binding to K562 target cells are predominantly Leu-11a$^+$ (Kang et al., 1987b).

Most previous studies on the ultrastructure and cytochemistry of human NK cells were performed on Percoll-purified LGL (Huhn et al., 1982; Grossi et al., 1982; Babcock and Phillips, 1983; Ferrarini and Grossi, 1986). However, reports have shown that only 90% of the Percoll-purified LGL express Leu-11a$^+$ phenotype (Phillips and Babcock, 1983) which is known to be a surface marker for functional human NK cells (Lanier et al., 1983, 1984; Kang et al., 1987b). In addition, anti-Leu-11 antibody reacts with virtually all the NK cells which express anti-tumor cytotoxic activity (Phillips and Babcock, 1983). Therefore, immunoelectron microscopic labeling methods for NK cell identification using anti-Leu-11 antibody are believed to be the most reliable means to study the ultrastructure and cytochemistry of human NK cells (Kang et al., 1987a,b).

Lymphokines including interferons (IFN) and interleukin-2 (IL-2) have been shown to augment the cytotoxicity of NK cells (Djeu et al., 1982; Ortaldo et al., 1984; Svedersky et al., 1984; Weigent et al., 1983). Studies have also indicated that the proliferation of NK cells is IL-2 dependent (Smith, 1984; Hefeneider et al., 1983; Domzig et al., 1983). A recent report shows that interleukin-4 (B cell stimulatory factor 1) induces lymphokine activated killer (LAK) cell activity and augments this activity in combination with IL-2 (Mule et al., 1987). In addition, the bacterial endotoxin (lipopolysaccharide, LPS), a potent immune regulator, has been shown to enhance NK cytotoxicity (Fink et al., 1984; Gangemi et al., 1980; Nowotny, 1985; Kang, et al., 1988). However, the ultrastructural and cytochemical aspects of the mechanisms by which LPS and IL-2 exert their effect on the enhancement of human NK activity are unclear. The present paper reviews the immunoelectron microscopic identification of human NK cells and reports the effects of LPS and IL-2 on the ultrastructure of Leu-11a$^+$ NK cells as well as the correlation of these effects with functional changes in NK activity.

Materials and Methods

Cell Preparation

Peripheral blood mononuclear cells (PBMC) were obtained by Ficoll-Hypaque centrifugation of heparinized peripheral venous blood from healthy volunteer donors (Boyum, 1968). Leu-11a$^+$ cells were isolated from PBMC by a FACS II cell sorter using a monoclonal anti-Leu-11a antibody conjugated with FITC (Becton Dickinson Monoclonal Center, Mountain View, CA) according to the method described by Biddison et al. (1981).

Treatment of Cells with LPS and IL-2

PBMC were suspended at a concentration of 1 x 10^6 cells per ml in RPMI 1640 supplemented with 10% heat-inactivated pooled human AB serum (Flow Laboratories, McLean, VA), 1% glutamine (Gibco Laboratories, Grand Island, NY), 1% penicillin/streptomycin and 2-mercaptoethanol at a concentration of 5 x 10^{-5} M. Cells were incubated in tissue culture flasks (type 25100, Corning Glass Works, Corning, NY) containing a total of 10 ml medium with 10, 50, and 100 µg/ml LPS in a humidified atmosphere containing 5% CO_2 in air for 24 h. The controls were cultured under the same conditions in the absence of LPS. Cells were harvested for ultrastructural and cytochemical examinations, and cytotoxicity assays 24 h after incubation. Culture supernatants were collected for interferon assays.

Purified Leu-11a$^+$ cells were treated with 50 µg/ml LPS in the same manner in microtiter plates at 37°C for 24 h. Cells were harvested for cytotoxicity assays after incubation.

PBMC were also incubated with recombinant IL-2 (IL-2) (Cetus Corporation, Emeryville, CA) at a concentration of 500 International Units (IU) per ml in the same conditions as above for 4 and 7 days. Cells were harvested for immunoelectron microscopic examination and cytotoxicity assay after incubation.

Phagocytosis

PBMC were incubated with opsonized heat-killed Staphylococcus aureus in culture flasks at 37°C for 4 h. Cells were then labeled with anti-Leu-11a antibody and processed for immunoelectron microscopy.

Inmmunoelectron Microscopy

Reagents. Mouse monoclonal antibodies (MoAB) against Leu-7 (HNK-1), Leu-11 (NKP-15) and Leu-19 (NKH-1) surface antigens of human NK cells were obtained from Becton Dickinson Monoclonal Center, Inc. (Mountain View, CA). A Vector ABC kit containing a biotinylated anti-mouse IgG, avidin D, and biotinylated horseradish peroxidase (HRP) was purchased from Vector Laboratories, Inc. (Burlingame, CA). Goat anti-mouse IgG or IgM antibody conjugated with 10, 20, or 40 nm colloidal gold (GAMG10, GAMG20, GAMG40) was procured from Janssen Pharmaceutical, Inc. (Piscataway, NJ). The specificity of all monoclonal antibodies used in this study are presented in Table 1.

Labeling with Antibodies. All PBMC samples from different experiments were incubated, respectively, with biotinylated anti-Leu-7, FITC conjugated anti-Leu-11a, and anti-Leu-19 in RPMI 1640 medium in ice for 60 min. Cells were fixed in suspension in 1% glutaraldehyde/1% paraformaldehyde in ice for 20 min (Kang et al., 1985). Biotin-conjugated anti-Leu-7 antibody was stained directly by an ABC procedure using HRP as an electron-dense marker (Kang et al., 1985). Anti-Leu-11a and anti-Leu-19 antibodies were visualized by the ABC method via a biotinylated anti-mouse IgG or by colloidal gold linked anti-mouse IgG (Kang et al., 1987a).

Cell samples prelabeled with anti-Leu-11a or anti-Leu-19 antibody were also incubated with carboxylate beads (1 µm size) coated with anti-mouse IgG (Polysciences, Inc., Warrington, PA) or with 40 nm gold linked anti-mouse IgG in ice for 30-45 min prior to fixation in 1% glutaraldehyde/1% paraformaldehyde for 20 min. After fixation and washing, cells labeled with antibody-coated beads were incubated with 10 nm gold-linked anti-mouse IgG as described previously (Kang et al., 1985). The samples were processed for both scanning (SEM) and transmission (TEM) electron microscopy.

Table 1. Specificity of monoclonal antibodies

Monoclonal antibody	Specificity
Leu-7	HNK-1 antigen, large granular lymphocytes and subset of NK cells.
Leu-11a (CD 16)	NKP-15 antigen, Fc receptor of large granular lymphocytes, NK cells and neutrophils.
Leu-19	NKH-1 antigen, IL-2 dependent clones, NK cells, but not neutrophils.

Dual Labeling with Antibodies. A peroxidase-colloidal gold double labeling procedure was used to identify NK cells displaying a combination of Leu-7 and Leu-11a antigens. PBMC were incubated with anti-Leu-11a (IgG immunoglobulin) after 3 washings in the RPMI 1640 medium. Anti-Leu-7 antibody was stained by 10 nm colloidal gold linked anti-mouse IgM in ice for 60 min immediately after incubation with the primary antibody. Cells were then fixed in suspension with 1% glutaraldehyde/1% paraformaldehyde in ice for 20 min. Anti-Leu-11a antibody was stained by HRP using the ABC method via a biotinylated anti-mouse IgG (Kang et al., 1987a).

Labeling of Effector-Target Conjugates with Anti-Leu-11a or Anti-Leu-7. For determination of the phenotypes of functional NK cells, effector-target conjugates were prepared by incubation of PBMC with K562 targets at 50:1 ratio in RPMI 1640 medium at 37°C for 60 min and then reacted, respectively, with anti-Leu-11a and anti-Leu-7 according to the procedures reported previously (Kang et al., 1987b).

Enzyme Localization in Leu-11a$^+$ Cells

In order to examine the effect of LPS and IL-2 on the activity of acid phosphatase (AcPase) or Ca^{2+}-ATPase in Leu-11a$^+$ cells, cells exposed to LPS for 24 h or treated with IL-2 for 4 days were labeled with anti-Leu-11a and 10 nm gold-conjugated anti-mouse IgG, and then processed for localization of enzymes according to the following methods.

AcPase. Cells treated with LPS for 24 h prelabeled with the specific antibodies were fixed in 2% glutaraldehyde/1% paraformaldehyde at 4°C for 30 min. After overnight washing in 0.1 M sodium cacodylate buffer (pH 7.2) in a refrigerator, cells were processed for AcPase localization by the method reported earlier (Kang et al., 1985).

Ca^{2+}-ATPase. Cells treated with LPS for 60 min or 24 h and cells treated with IL-2 for 4 days were used for the enzyme localization. After labeling with anti-Leu-11a and anti-mouse IgG conjugated to 10 nm gold, cells were fixed in 3% paraformaldehyde at 4°C for 30 min. Following overnight washing in sodium cacodylate buffer in a refrigerator, samples were processed for localization of Ca^{2+}-ATPase by a technique modified from the method reported by Ando et al., (1981). Disodium salt of ATP (Sigma Chemical Co) was used as a specific substrate and cerium chloride (Sigma) was used to capture the reaction product of the enzyme activity.

Electron Microscopy

All samples were postfixed in 2% osmium tetroxide at 4°C for 2 hr, dehydrated in a series of graded ethanol solutions, and embedded in PolyBed (Polysciences, Warrington, PA). Ultrathin sections prepared with a diamond knife were briefly stained in lead citrate, and examined in a JEOL 100 CX TEM. For SEM, cells were monolayered on polylysine coated cover glasses, dehydrated in a series of graded ethanol solutions, dried in liquid CO_2, shadowed with gold and palladium or with a thin layer of carbon only for cells labeled with colloidal gold (GAMG40) and examined in a JEOL JSM 35CF SEM.

The overall procedure for immunoelectron microscopy is summarized in Figure 1.

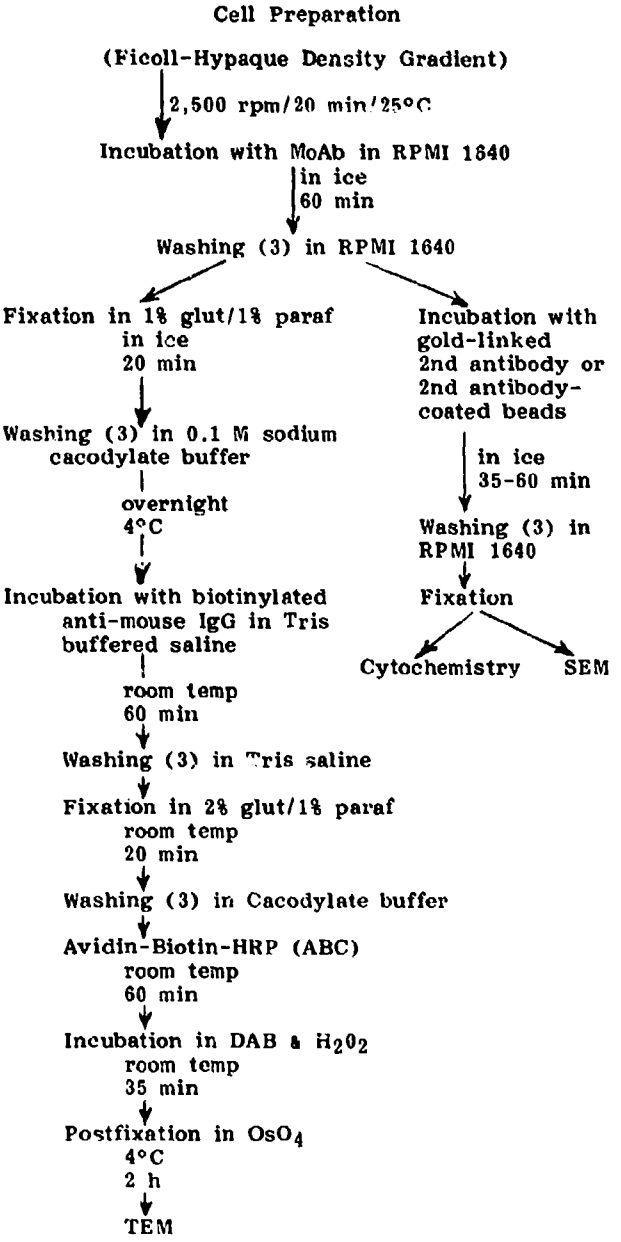
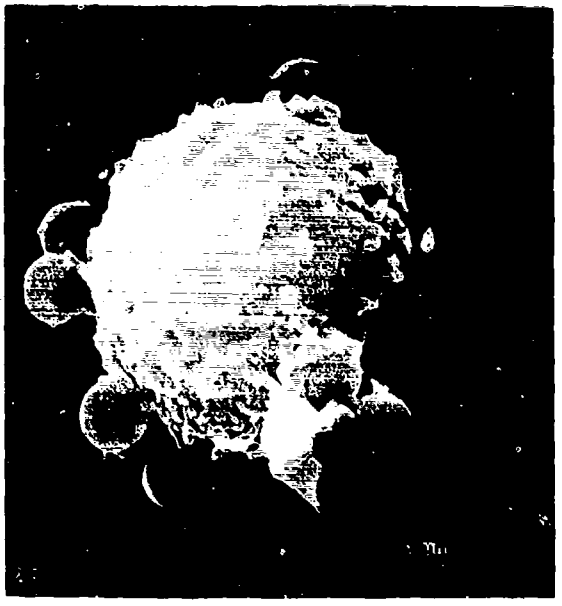
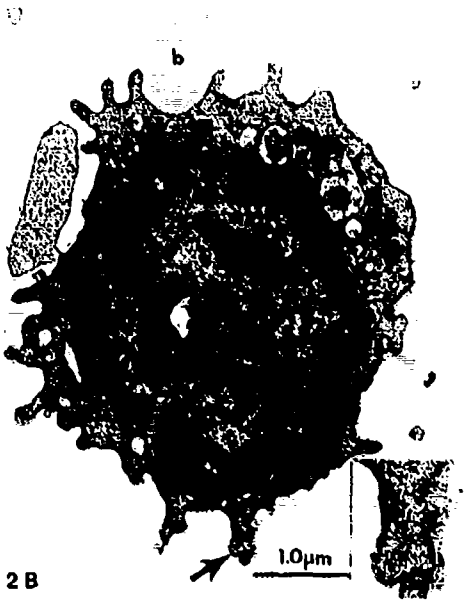


Fig. 1. Summary of the procedure for immunoelectron microscopic labeling of human NK cells using immunogold and immunoperoxidase techniques.





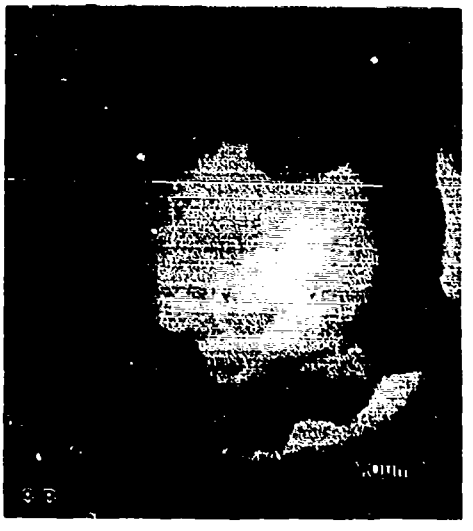


Fig. 2. PBMC labeled with anti-Leu-11a, and visualized by carboxylate beads coated with anti-mouse IgG, and 10 nm gold linked anti-mouse IgG. A. Scanning electron micrograph of a Leu-11a$^+$ cell shows binding of beads to the cell surface. B. Transmission electron micrograph of a Leu-11a$^+$ cell labeled by the bead (b) and 10 nm gold grains on the cell surface. Inset indicates a magnified cytoplasmic projection (arrow) revealing gold labeling.

Fig. 3. PBMC labeled with anti-Leu-19 antibody and 40 nm gold conjugated anti-mouse IgG, and processed for SEM. A. Using back-scattered electron image (BEI) combined with secondary electron image (SEI) technique, the Leu-19$^+$ cell is identified by numerous gold grains (white dots) on the cell surface. B. The same micrograph of Fig. 3A demonstrated by BEI in reverse polarity. Black dots on the cell surface are gold grains.

Statistical Evaluation.

The changes in the number of electron-dense granules in Leu-7$^+$ cells and Leu-11a$^+$ cells 4 and 7 days after stimulation with IL-2 was assessed by counting the granules in an ultrathin section of a cell which was sectioned through the nuclear plane. At least 50 cells were evaluated. The number of granules per cell section was expressed as mean ± standard error.

Assay for NK Cytotoxicity

Effector cells were obtained from PBMC which had been washed with RPMI 1640 after incubation with LPS or IL-2. K562 myeloid cells (American Type Culture Collection, Rockville, MD) were used as target cells for cytolytic assays. Cytotoxicity assays were performed in 96-well v-bottom microtiter plates (PGC Scientific, Gaithersburg, MD), and each effect-

or: target (E/T) ratio was performed in triplicate. Target cells were radiolabeled with 240 μCi of $Na^{51}CrO_4$ for 60 to 90 min at 37°C, washed 3 times and viable target cells in 50 microliters of medium were added to varying numbers of effector cells (in 100 microliters of medium). After incubating in the microliter plates for 4 to 6 h at 37°C, 50 microliters of supernatant were removed from each well. In addition, each assay contained target cells incubated with medium alone in the absence of added effector cells (spontaneous release) and target cells incubated in 5% Triton X-100 (maximum release). Percent specific cytotoxicity was calculated as follows:

$$\text{Percent specific lysis} = \frac{\text{Experimental release (CPM)} - \text{Spontaneous release (CPM)}}{\text{Maximum release (CPM)} - \text{Spontaneous release (CPM)}} \times 100$$

The cytotoxicity of the purified Leu-11a$^+$ cells after 24 h incubation with LPS was assessed by the same procedure. A duplicate assay was performed 5 weeks later.

Interferon Assays

The supernatant of the culture medium was collected 24 h following incubation with various doses of LPS. Total interferon was assayed in human KB cells as previously reported (Maheshwari and Friedman, 1980). The titers of interferon were determined against an international standard of human gamma interferon from NIAID, NIH, Bethesda, MD.

Results

Identification of Leu-11a$^+$ and Leu-19$^+$ NK Cells by SEM

By SEM, Leu-11a$^+$ cells were identified by binding of many anti-mouse IgG coated 1 μm beads on the cell surface which had numerous microvilli with various lengths (Fig. 2A). TEM of the same cell sample also revealed that Leu-11a$^+$ cells were bound by the beads and gold grains on the cell surface (Fig. 2B). By SEM Leu-19$^+$ NK cells could also be recognized by the presence of numerous gold grains on the cell surface (Figs. 3A,B).

Ultrastructure of NK Cells

Leu-7$^+$/Leu-11a$^-$ Cells. Cells of this subset were identified by gold labeling on the cell surface. These cells displayed a smooth cell surface with a few cytoplasmic projections, an irregular or reniform nucleus, Golgi complex, simple rough endoplasmic reticulum (RER), mitochondria, small vesicles, and multivesicular bodies. Electron-dense granules, parallel tubular arrays (PTA), and paracrystalline inclusions were not observed in this subset. The cells had high nucleocytoplasmic (N/C) ratios (Kang et al., 1987a).

Leu-7$^+$/Leu-11a$^+$ Cells. The majority of human NK cells in the PBL expressed a positive combination of Leu-7 and Leu-11a antigens. These cells were stained by both HRP reaction product and colloidal gold on the cell surface (Fig. 4). This NK subset had a low N/C ratio (Kang et al., 1987a). The Leu-7$^+$/Leu-11a$^+$ cells had a reniform nucleus, well defined Golgi complex, centrioles, many electron-dense granules, RER, PTA, paracrystalline inclusions, multivesicular bodies, and numerous mitochondria (Fig. 5).

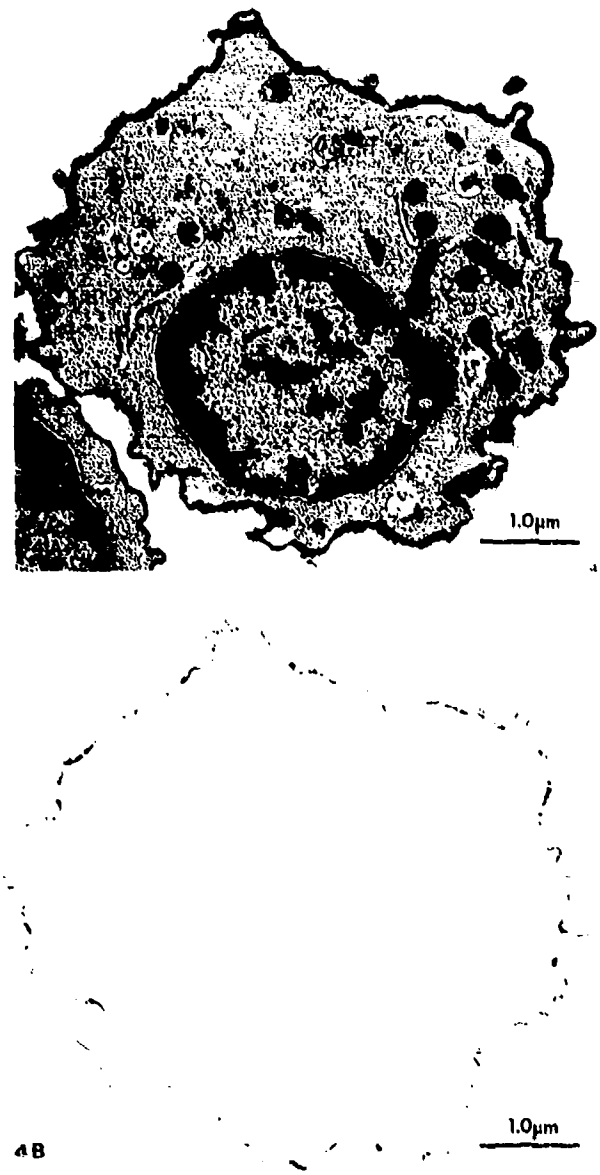


Fig. 4. PBMC processed by an immunogold and immunoperoxidase double labeling technique. A. Leu-7$^+$/Leu-11a$^+$ cell labeled by HRP reaction product for Leu-11a antigen and gold grains for Leu-7 antigen. B. Under-exposed micrograph of Fig. 4A to reveal gold grains on the cell surface.

Leu-7$^-$/Leu-11a$^+$ Cells. Leu-7$^-$/Leu-11a$^+$ cells were stained only by HRP reaction product on the cell surface. There were no distinct differences in the cytostructure between Leu-7$^+$/Leu-11a$^+$ cells and Leu-7$^-$/Leu-11a$^+$ cells (Kang et al., 1987a). The abundant cytoplasm contained all the major NK characteristic organelles such as electron-dense granules, PTA, paracrystalline inclusions, vacuoles, and Golgi complex (Fig. 6). The paracrystalline

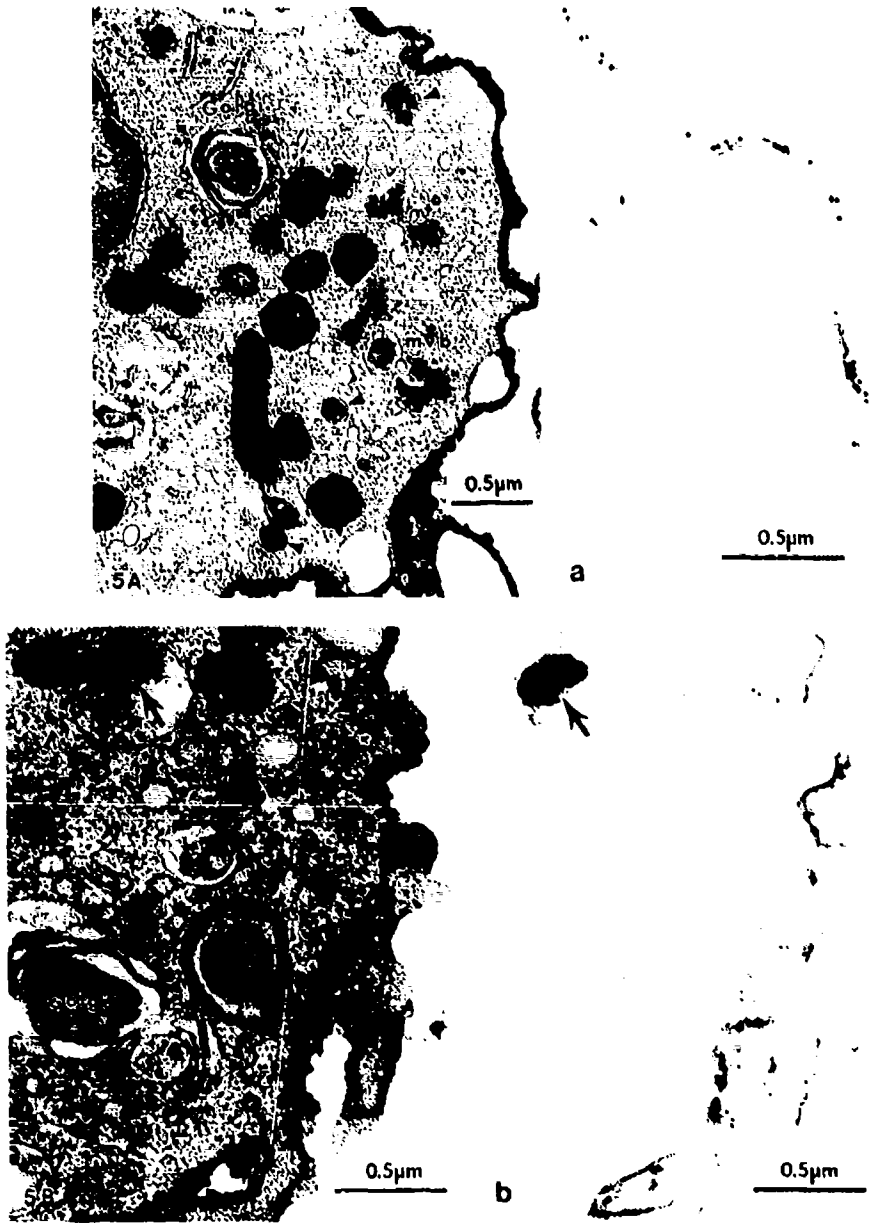


Fig. 5. PBMC processed by an immunogold and immunoperoxidase double labeling technique. Micrographs illustrate the ultrastructure of Leu-7$^+$/Leu-11a$^+$ cells.

A. Well-defined Golgi complex, electron-dense granules (arrow heads), a multivesicular body (mvb) and a centriole (c) are seen in the cell. Inset (a) is an under-exposed magnified micrograph of the same cell revealing gold grains on the cell surface.

B. Paracrystalline inclusion (arrow), many vesicles, and Golgi complex are shown in a Leu-7$^+$/Leu-11a$^+$ cell. Inset (b) is an under-exposed micrograph of the same cell to show the paracrystalline inclusion (arrow) and gold grains on the cell surface.

inclusions were often observed in association with PTA.

Leu-19$^+$ Cells. Ultrastructurally Leu-19$^+$ cells had a morphology resembling that of Leu-7$^+$/Leu-11a$^+$ and Leu-7$^-$/Leu-11a$^+$ cells. These cells had a reniform nucleus, a higher N/C ratio, villous cell surface, well defined Golgi complex, distinct RER, numerous electron-dense granules, mitochondria, and centrioles (Fig. 7). This subset represented approximately 16% of PBL.

Effector and Target Conjugates

We previously reported that most of the effector cells displaying "true" conjugation with target cells were Leu-11a$^+$ cells (Kang et al., 1987a). The Leu-11a$^+$ cells binding to K562 target were characterized by having a broad cell-to-cell contact with target cells by a shallow cytoplasmic interdigitation and polarization of cytoplasmic organelles toward target (Fig. 8). The polarized organelles included electron-dense granules, vacuoles with membranous materials or PTA, small vesicles, Golgi complex, and centrioles.

Phagocytosis

Ingested bacteria were observed in phagocytic vacuoles of Leu-11a$^+$ cells (Fig. 9) as reported previously (Kang et al., 1987b). In one case, 37% of the Leu-11a$^+$ cells from LPS-treated PBMC displayed ingestion of bacteria, while only 15% of Leu-11a$^+$ cells in the freshly isolated PBMC had bacteria (Kang et al., 1988).

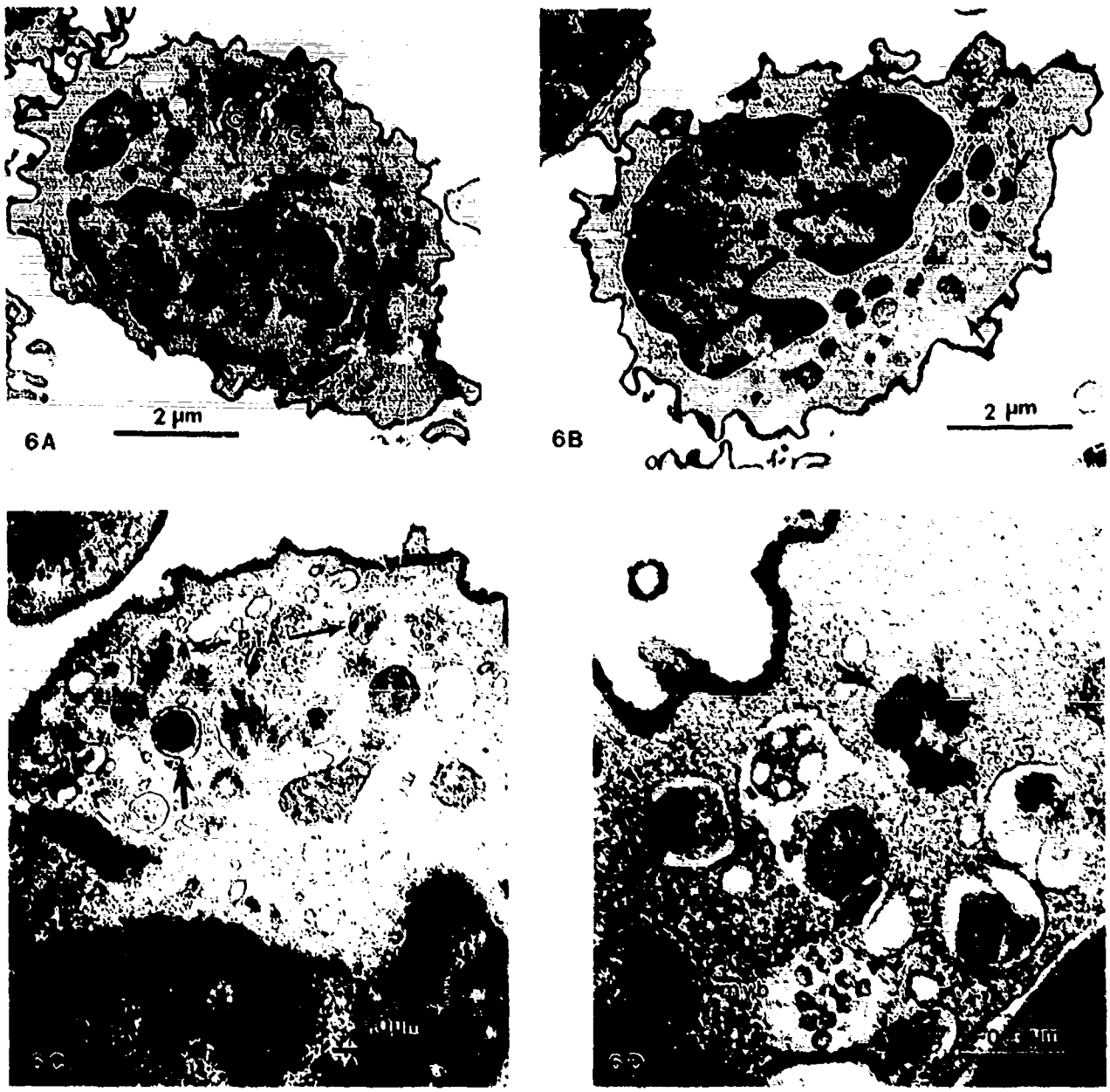


Fig. 6. Leu-7$^-$/Leu-11a$^+$ cells in PBMC labeled by an immunogold and immunoperoxidase method. A. Micrograph depicting the ultrastructure of a Leu-7$^-$/Leu-11a$^+$ cell which contains Golgi complex (G), a centriole (C), and vesicles with electron-dense material (arrows). B. Vacuoles (arrows) containing electron-dense or membranous materials are often observed in Leu-7$^-$/Leu-11a$^+$ cells. C. Membrane-bound electron-dense granule (arrow) and parallel tubular arrays (PTA) characteristics of Leu-7$^-$/Leu-11a$^+$ cell are shown in the micrograph. D. Paracrystalline inclusion (arrow), vacuole with electron-dense material (arrow head), and multivesicular body (MVB) are observed in Leu-7$^-$/Leu-11a$^+$ cells.

Effect of LPS and IL-2 on the Ultrastructure and Cytochemistry of Leu-11a$^+$ Cells

Some ultrastructural alterations were observed in Leu-11a$^+$ cells 24 h after incubation with LPS. The cisternae of RER, nuclear envelope, and Golgi saccules showed apparent distention (Fig. 10). Numerous small vesicles and many large electron-dense granules were often found in the cytoplasm of Leu-11a$^+$ cells treated with 100 μg/ml LPS which was the dose showing the most potent effect on the cytotoxicity. Tubuloreticular inclusions (TRI) were observed in the cisternae of RER in Leu-11a$^+$ cells treated with 50 or 100 μg/ml LPS for 24 h (Kang et al., 1988). Increased AcPase activity was also observed

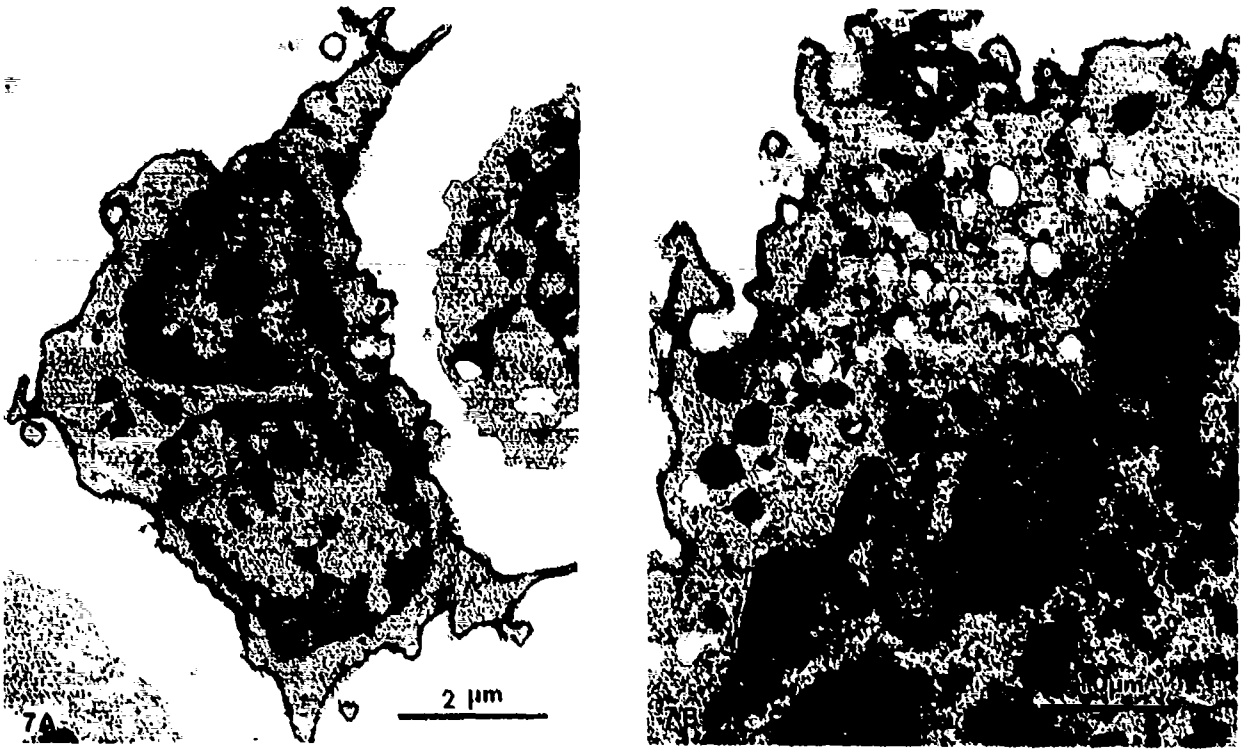


Fig. 7 (above). PBMC labeled with anti-Leu-19 and stained by HRP via anti-mouse IgG using the ABC method. A. Micrograph shows a Leu-19$^+$ cell stained by HRP reaction product. B. Higher magnification of a Leu-19$^+$ cell depicting numerous vesicles, electron-dense granules (arrows), and multivesicular bodies (mvb).

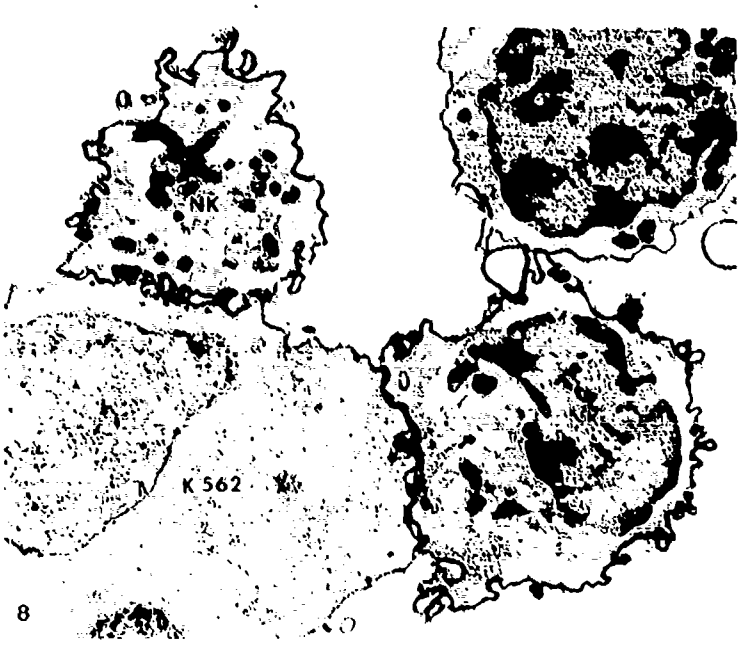
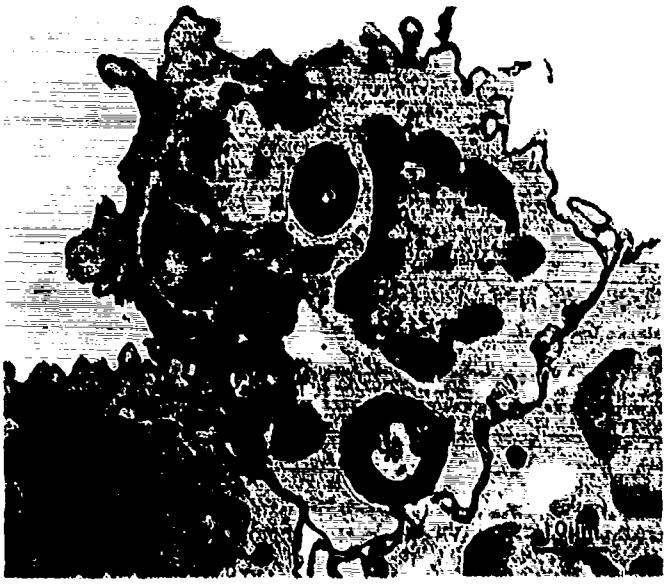
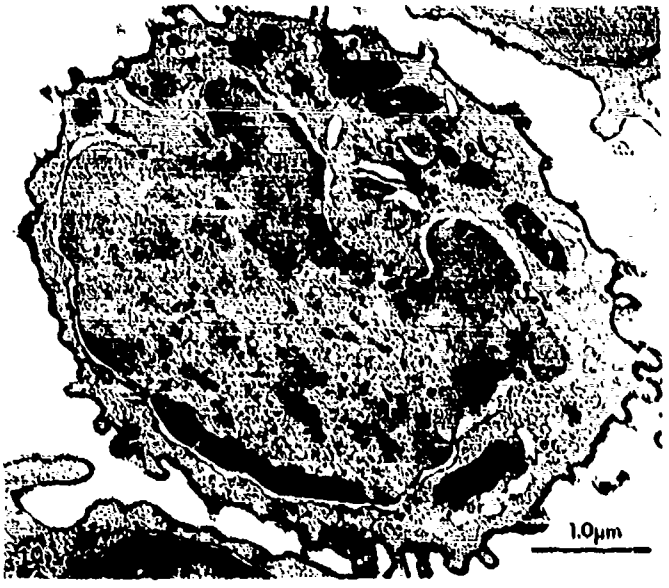


Fig. 9 (to the right). PBMC incubated with opsonized heat-killed Staphylococcus aureus and labeled with anti-Leu-11a antibody. Ingested bacteria (asterisk) are clearly seen in a Leu-11a$^+$ cell.

Fig. 10 (to the right). Leu-11a$^+$ cells exposed to LPS for 24 hr. The cell shows distinct dilation of the cisternae of Golgi saccules (G), nuclear envelope, and rough endoplasmic reticulum (er). Electron dense granules (arrows) are frequently observed in these cells.

Fig. 8. Effector-target conjugates labeled with anti-Leu-11a antibody and stained by HRP via anti-mouse IgG using the ABC method. Two Leu-11a$^+$ cells (NK) are attaching to a K562 target cell. Note the organelles of the NK cells are oriented toward the target cell.





in the same cells as compared to that of the control cells. As compared to the control Leu-11a$^+$ cells (Fig. 11) the Ca^{2+}-ATPase activity was distinctly decreased in the LPS-treated Leu-11a$^+$ cells (Fig. 12).

With respect to the ultrastructural effect of IL-2 on Leu-7$^+$ cells and Leu-11a$^+$ cells from IL-2 treated PBMC, profound changes were found 4 and 7 days after stimulation with the lymphokine. Many blastoid forms of Leu-7$^+$ cells and Leu-11a$^+$ cells were seen with an average maximal diameter of 10.00 ± 0.6 μm (Fig. 13A). Cells with mitotic figures were also often observed (Fig. 13B). Rough endoplasmic reticulum and Golgi complex were extensively hypertrophied in Leu-11a$^+$ cells particularly 7 days after incubation with IL-2 (Figs. 14A, B). The number of electron-dense granules in both Leu-7$^+$ cells and Leu-11a$^+$ cells increased significantly as compared to the non-stimulated control cells ($p < 0.001$). An average of 2.34 ± 1.0 and 2.87 ± 1.1 granules per cell section were, respectively, found in Leu-7$^+$ cells (Fig. 15) and Leu-11a$^+$ cells (Fig. 16) 4 days after stimulation with IL-2, while only 1.58 ± 1.0 granules per cell section were observed in non-IL-2 treated control samples. After 7-day treatment with IL-2, an average of 5.7 ± 2.5 granules per cell section was found in Leu-11a$^+$ cells (Fig. 17), whereas only 1.2 ± 0.8 granules per cell section were counted in the unstimulated Leu-11a$^+$ cells. TRI were also observed in the cisternae of RER of Leu-7$^+$ cells after stimulation with IL-2 for 4 days (Fig. 18). Higher Ca^{2+}-ATPase activity was observed in Leu-11a$^+$ cells stimulated with IL-2 for 4 days as compared to that of the non-stimulated Leu-11a$^+$ cells.

Enhancement of NK Activity and Interferon Production by LPS

Results from the cytotoxicity assays indicated 1.5- to 2-fold increases in the NK cytotoxicity of PBMC treated with LPS for 24 h as compared to the non-LPS treated controls (Table 2). In some cases, the increase in cytotoxicity correlated directly with an increase in LPS concentrations. There was a significant increase in NK activity in sorted Leu-11a$^+$ cells incubated with 50 μg/ml LPS for 24 h as compared to freshly isolated Leu-11a$^+$ cells ($p < 0.02$) or Leu-11a$^+$ cells incubated in vitro for 24 h in the absence of LPS ($p < 0.03$) (Table 3).

In parallel with NK cytotoxicity, the total interferon levels in the supernatants of the LPS-treated PBMC showed a significant dose-dependent increase with LPS concentrations (Table 2).

Table 2. Percent of NK specific lysis of K562 cells following 24 hr exposure of PBMC to varying concentrations of LPS from E. coli

	%Killing E/T ratio		Interferon (IU/ml)
	50:1	12:1	
Control	41.92±3.5	18.09±1.1	0
10 μg/ml LPS	54.11±1.2	24.73±4	13
50	71.36±5.1	43.34±3.4	50
100	100±0.7	51.33±0.9	150

Enhancement of NK Activity by IL-2

A significantly higher percentage of target cells were killed by effectors stimulated with IL-2 for 4 or 7 days as compared to that of the controls ($p < 0.05$) (Table 4). As seen in Table 4, at least a 10-fold increase in the cytotoxicity was observed 4 days after stimulation with IL-2.

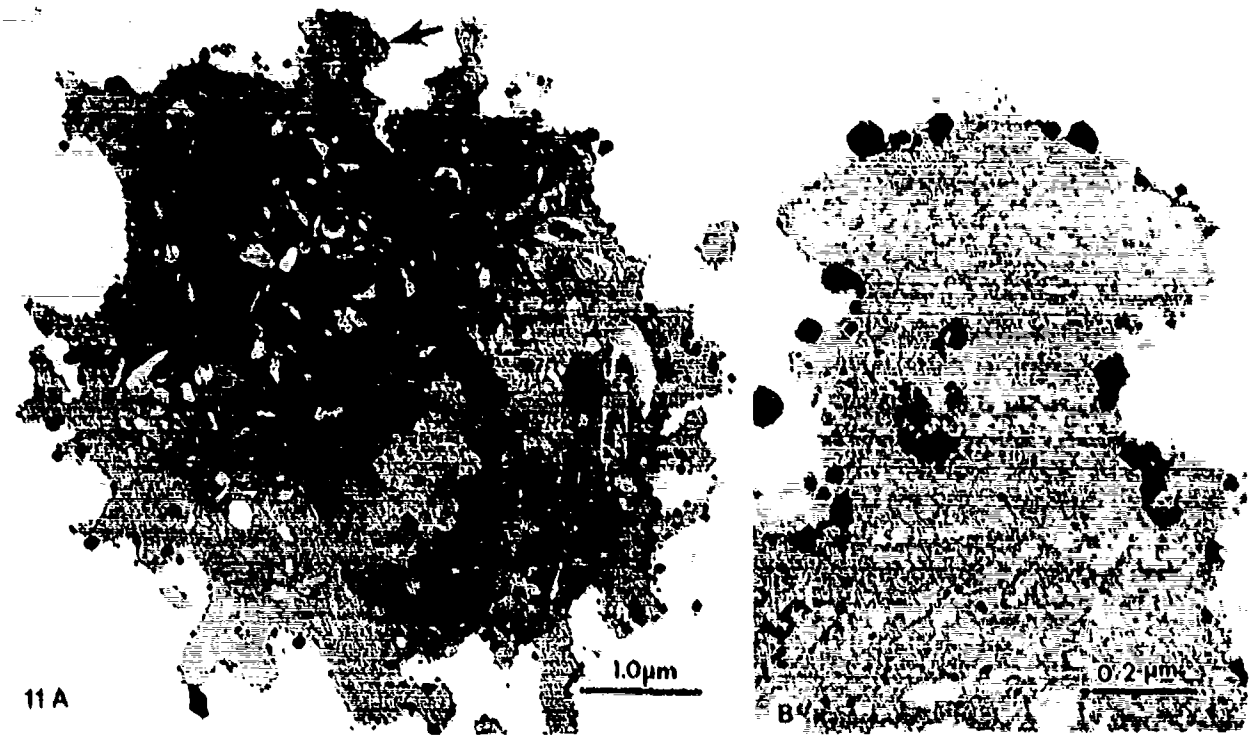


Fig. 11. Leu-11a$^+$ cell of the normal control sample processed for Ca^{2+}-ATPase localization. A. Reaction product of the enzyme activity is uniformly distributed throughout the cell surface. B. A higher magnification of a small portion (arrow in fig. 11A) of the same cell reveals gold grains on the cell surface.
Fig. 12. Leu-11a$^+$ cells exposed to LPS for 60 min and processed for Ca^{2+}-ATPase localization. A. Micrograph shows weak staining for the enzyme activity on the cell surface of a Leu-11a$^+$ cell. B. High magnification of a small portion (arrow in fig. 12A) of the same cell showing gold grains and the enzyme reaction product on the cell surface.

Table 3. Percent of NK specific lysis of K562 cells in sorted Leu-11a$^+$ cells following 24 h incubation with 50 ug/ml LPS

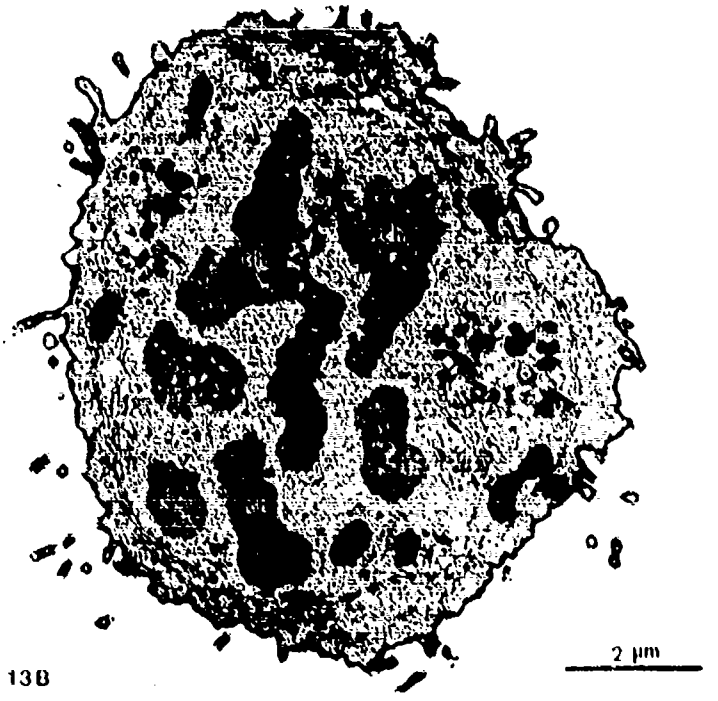
	Specific Cytotoxicity (%)*	
	Expt. 1	Expt. 2
Day 0 (prior to incubation)	56.6±4.4	N.D.
24 h incubation without LPS	56.1±1.6	56.5±3.3
24 h incubation with LPS	70.1±5.8	65.1±1.7

*E/T ratio, 20:1; N.D., not done

Discussion

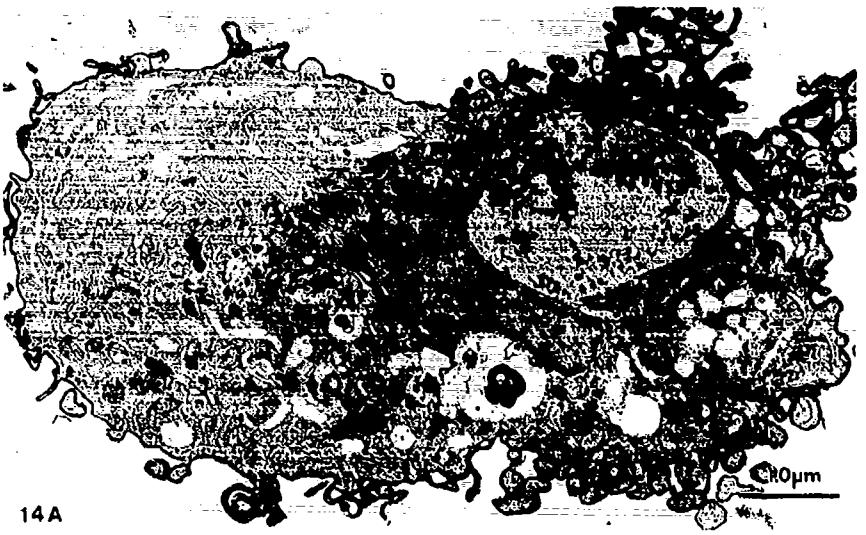
Accurate cell identification is a prerequisite for study of the influence of LPS and lymphokines on the ultrastructure and cytochemistry of human NK cells. We have established a technique for immunoelectron microscopic identification of human NK cells using immunoperoxidase and immunogold systems and have found that the prefixation method by incubation of live cells with a specific primary antibody prior to fixation is the most effective and reliable technique for labeling human NK cells expressing Leu-7, Leu-11, and Leu-19 surface antigens (Kang et al., 1985, 1987a,b). Manara and his coworkers (1984, 1985, 1986a,b) have employed a postfixation method to label human NK cells with Leu-7 and Leu-11a surface antigens. Indeed, the antigenicity of Leu-7 antigen can be preserved following a brief fixation in 1% glutaraldehyde/1% paraformaldehyde (Kang et al., 1984, 1985). However, the antigenicity of Leu-11a and Leu-19 is often diminished or totally abrogated following such fixation (Kang et al., 1985). The Leu-11a$^+$ cells presented by Manara (1985, 1986a) in several papers are more similar to the size and ultrastructure of human monocytes than the Leu-11a$^+$ cells which were identified by our established prefixation labeling method. In fact, human monocytes have also been shown weakly stained by anti-Leu-11a antibody (Kang et al., 1985). In addition, the HRP

13A

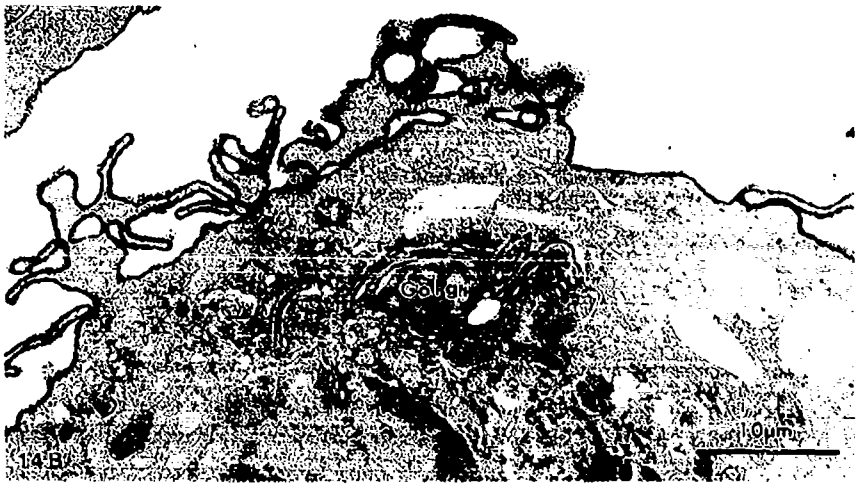


13B

Fig. 13 (above). PBMC incubated with IL-2 for 4 to 7 days and labeled with anti-Leu-11a antibody. A. Micrograph shows a large IL-2 stimulated blastoid Leu-11a$^+$ cell and a small resting Leu-11a$^+$ cell. B. Large Leu-11a$^+$ cells with chromosomes (Ch), distinct rough endoplasmic reticulum, and electron-dense granules are often observed in PBMC 4 days after IL-2 stimulation.



14A



14B

Fig. 14. PBMC exposed to IL-2 for 7 days and stained by anti-Leu-11a antibody.

A. Micrograph shows highly hypertrophied rough endoplasmic reticulum in a Leu-11a$^+$ cell.

B. Golgi complex is also hypertrophied in Leu-11a$^+$ cells.

Table 4. Percent of NK specific lysis of K562 cells in PBMC following exposure to 500 iU IL-2 for 4 and 7 days

	% Specific Lysis		
Donor 1	E/T ratio		
	25:1	12:1	6:1
Day 0	6.2±0.1	4.7±0.1	1.5±0.1
Day 4	63±0.3	63.2±0.1	62.6±0.2
Donor 2			
	50:1	25:1	12:1
Day 0	73.2±2.4	54.2±8.0	50.1±3.5
Day 7	92.5±6.9	88.3±0.2	75.3±3.8

staining on the cell surface of Leu-7$^+$/Leu-11a$^+$ cells shown by Manara et al. (1985, 1986a,b) is possibly a stain produced by osmium and ferrocyanide (OsFeCN) which was used for postfixation by Manara's group. Utilization of OsFeCN as a second fixative in immunocytochemistry can be a pitfall since OsFeCN has been shown to stain glycocalyx of muscle cells (Forbes et al., 1977) and lymphocytes (our unpublished observations).

The Leu-7$^+$/Leu-11a$^-$ cells display a closer ultrastructural similarity to human T cells (Kang et al., 1987a) and have been reported to be an immature form of NK cells (Manara et al., 1985). This subset is known to be the least effective NK cell in human PBL (Lanier et al., 1983). As compared to Leu-11a$^+$ cells, ultrastructurally Leu-19$^+$ cells seem to show a closer relationship to Leu-7$^+$/Leu-11a$^+$ and Leu-7$^-$/Leu-11a$^+$ cells. These morphological differences may reflect the variations in NK capacity among NK subsets. The Leu-11a$^+$ cells identified by anti-Leu-11a alone in the LPS and IL-2 treated samples could be either Leu-7$^+$/Leu-11a$^+$ or Leu-7$^-$/Leu-11a$^+$ cells according to their ultrastructural characteristics.

The lineage of NK cells remains uncertain. NK cells have been traditionally described as non-phagocytic lymphoid cells that mediate spontaneous cytotoxicity against tumor target (Grossi et al., 1982; Huhn et al., 1982). However, other investigators have demonstrated ingestion of bacteria by LGL (Babcock and Phillips, 1983) and ingestion of bacteria by lymphocytes containing PTA (Payne and Nagle, 1980) which are thought to be a marker structure of human NK cells (Payne and Glasser, 1981; Burns et al., 1982; Zucker-Franklin et al., 1983). Our observations of endogenous peroxidase (Kang et al., 1987a) and phagocytic activity (Kang et al., 1987b, 1988) in Leu-11a$^+$ cells provide additional evidence supporting the notion that the human NK cell is a "phagocyte in lymphocyte's clothing" (Babior and Parkinson, 1982).

The membrane-bound electron-dense granules are classically described as the main ultrastructural characteristics of NK cells. They contain glycoprotein, lysosomal enzymes including acid phosphatase, arylsulfatase (Kang et al., 1987a; Zucker-Franklin et al., 1983), and trimetaphosphatase (Frey et al., 1982), perforins (Henkart, 1985) and serine esterases (Young et al., 1986). Thus, the electron-dense granules are believed to be involved in the process of NK cell-mediated cytolysis (Neighbour et al., 1982; Nocera et al., 1983; Grossi et al., 1982; Babcock and Phillips, 1983; Zucker-Franklin et al., 1983; Carpen et al., 1981; Frey et al., 1982). Blockage of the secretion of these cytoplasmic granules by monensin (Carpen et al., 1982) or induction of the secretion of these granules by strontium prior to binding to target cells (Neighbour et al., 1982) impairs NK activity. In the present study, we have observed a significant increase in the number of the electron-dense granules in Leu-11a$^+$ cells after exposure to IL-2 for 4 or 7 days. This increase shows a positive correlation with the IL-2 enhanced NK activity.

PTA have been consistently observed in Leu-7$^+$ cells and Leu-11a$^+$ cells (Kang et al., 1985, 1987a,b, 1988; Manara et al., 1984, 1985) as previously found in LGL (Huhn et al., 1982; Zucker-Franklin et al., 1983; Payne and Glasser, 1981; Payne et al., 1983; Payne, 1984; Henkart and Henkart, 1982). Therefore, this unique organelle has been considered as a marker structure of human NK cells (Babcock and Phillips, 1983). Cytochemically PTA have been shown to contain acid phosphatase, arylsulfatase, and glycoprotein (Kang et al., 1987a). The presence of lysosomal enzymes in PTA and the close association of PTA with paracrystalline inclusions suggest that the latter is possibly derived from PTA (Kang et al., 1987a). The function of PTA remains uncertain. Reports have indicated that PTA may be involved in induction of membrane lesions of target cells (Zucker-Franklin et al., 1983; Henkart et al., 1982; Podack and Dennert, 1983; Tschopp et al., 1982; Dennert and Podack, 1983). A recent report shows that the paracrystalline inclusions in human NK clones are possibly involved in cytolysis of target cells (Caulfield et al., 1987). However, we have not observed PTA or paracrystalline inclusions in Leu-11a$^+$ cells following in vitro incubation with or without IL-2 for 4 to 7 days. Whether this structure is related to NK cytotoxicity requires further investigation.

Elaboration of Golgi complex and rough endoplasmic reticulum in Leu-11a$^+$ cells following exposure to LPS suggests active synthesis of new materials possibly for fabrication of electron-dense granules

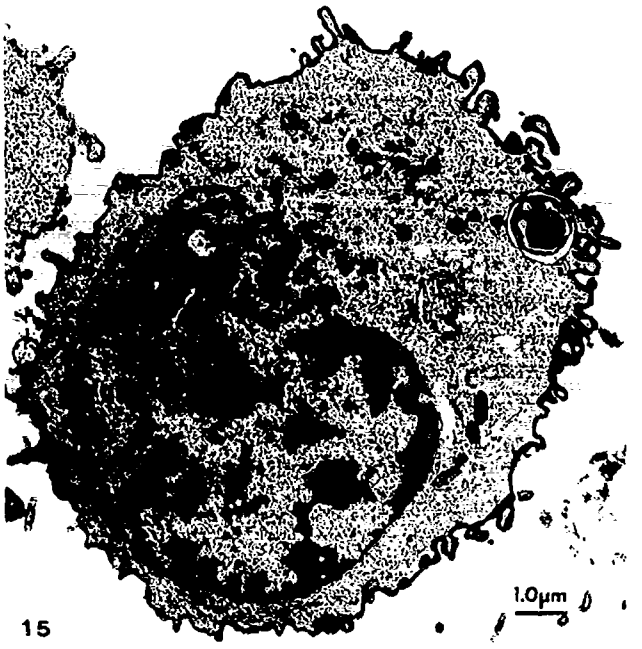


Fig. 15. PBMC exposed to IL-2 for 4 days and labeled with anti-Leu-7 antibody and stained by HRP using the ABC methods. Numerous electron-dense granules are observed in Leu-7$^+$ cells.

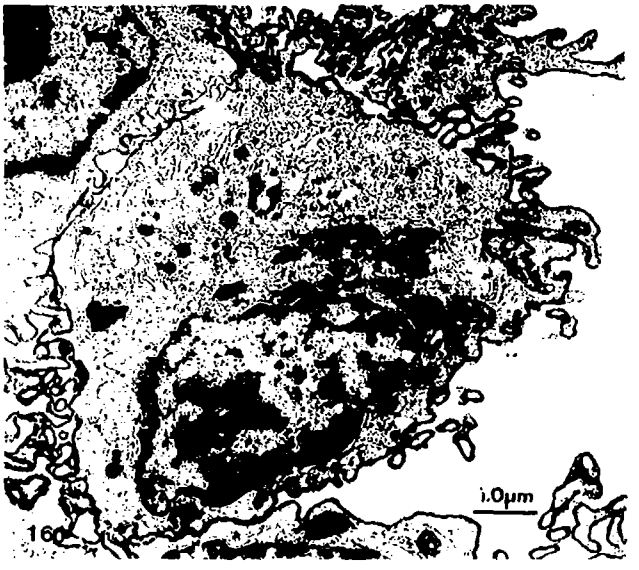


Fig. 16. PBMC stimulated by IL-2 for 4 days and labeled with anti-Leu-11a antibody. Many electron-dense granules are present in the Leu-11a$^+$ cell.

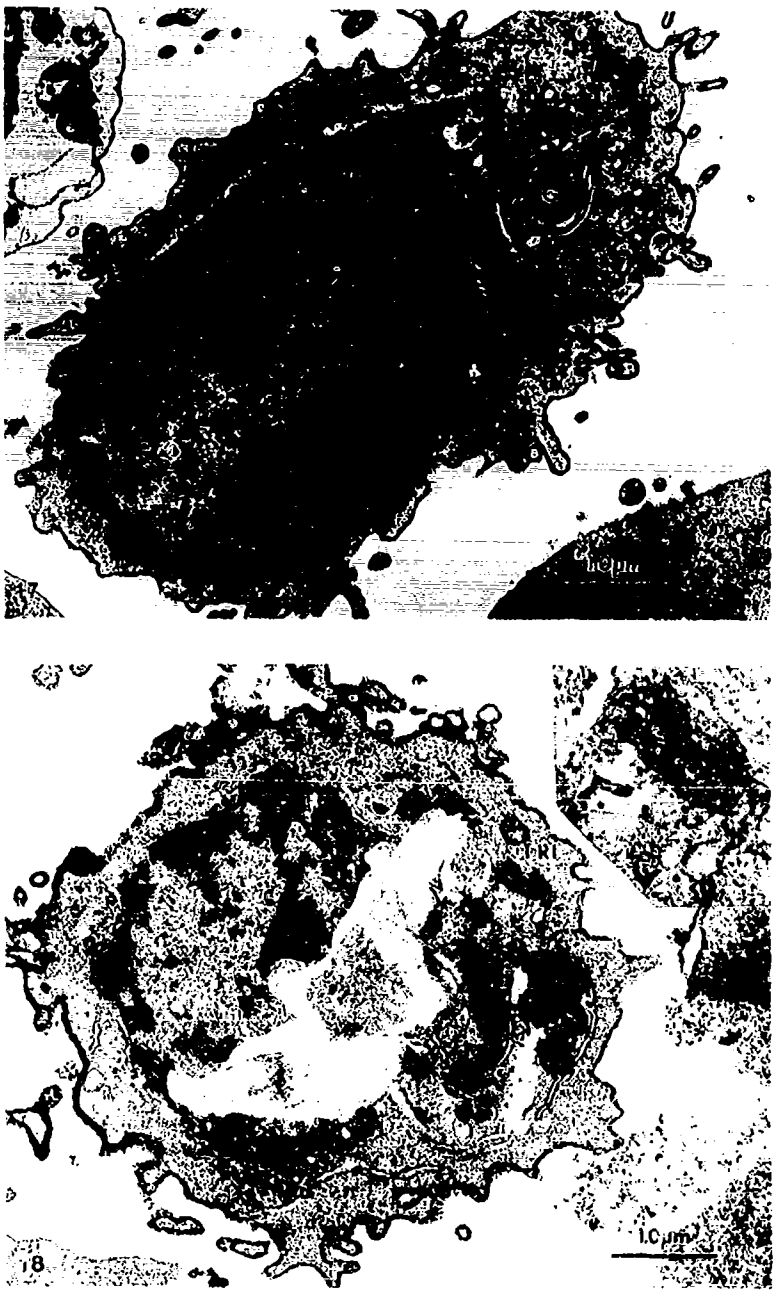


Fig. 17. PBMC exposed to IL-2 for 7 days and labeled with anti-Leu-11a antibody. The number of electron-dense granules is significantly increased after 7-day exposure to IL-2. The Golgi complex (G) is also elaborated.

Fig. 18. Leu-7$^+$ cells exposed to IL-2 for 4 days. A TRI is observed in the cisterna of rough endoplasmic reticulum of a Leu-7$^+$ cell. Inset higher magnification of the TRI.

(Farquar, 1985) or for production of IFN (Djeu et al., 1982). In fact, increased acid phosphatase activity was observed in Leu-11a$^+$ cells which were exposed to higher doses of LPS. An increase in the size and number of electron-dense granules may facilitate the lytic ability of NK cells. Whether the products of LPS stimulation in human PBMC may also increase binding of effector cells to target cells remains unknown. We have observed that IL-2 significantly increased the binding of Leu-11a$^+$ cells to K562 targets (unpublished data).

The inhibition of Ca^{2+}-ATPase activity by LPS in Leu-11a$^+$ cells may result in an increase of intracellular Ca^{2+} (Carafoli and Longeni, 1986). Ca^{2+} is known to be required in reorganization of microtubules and reorientation of cytoplasmic organelles in NK cells during the process of cytolysis (Henkart, 1985). An increase of intracellular Ca^{2+} may activate the lytic program of NK cells (Anasetti et al., 1987).

It is well established that interferons (IFN) including gamma interferon (IFN-r) activate NK cell mediated cytotoxicity against tumor cells (Herberman et al., 1979; Lucero et al., 1981; Brunda and Davatelis, 1985). In the present study, the positive correlation of increased NK cytotoxicity with production of IFN suggests that IFN is related to the enhancement of NK cytotoxicity in PBMC treated with LPS. The effect of IFN on NK cells is also indicated by the formation of TRI in Leu-11a$^+$ cells. TRI are a proven marker of IFN stimulation in human PBL (Grimley et al., 1985). Reports have shown that LPS induces production of alpha and beta interferons in macrophages and B cells (Maehara and Ho, 1977; Havell and Spitalny, 1983). However, production of IFN-r by stimulation with LPS in T cells and NK cells requires activation from IL-2 or macrophages (Blanchard et al., 1986; Matsumura and Nakano, 1988). Recently, Matsumura and Nakano (1988) reported that a direct contact of IFN-r producing cells with macrophages is essential to LPS-induced production of the interferon. Indeed, the intimate ultrastructural association of human Leu-11$^+$ NK cells with monocytes has been observed in human PBMC (Kang et al., 1987b). Studies have shown that LGL produce IFN-r following IL-2 stimulation (Trinchieri et al., 1984; Ortaldo et al., 1984; Young and Ortaldo, 1987). In this regard, IL-2 has been believed to be required for the production of IFN-r by T cells or LGL (Handa et al., 1983). Although LPS is a poor direct inducer for IL-2 production (Le et al., 1986), LPS may indirectly induce production of IL-2 by T cells (Simon and Lee,

1985) and LGL (Pistoia et al., 1983) via stimulation with IL-1 which is produced by macrophages / monocytes following LPS stimulation (Arend et al., 1985; Dinarello, 1985; Haeffner-Cavaillon et al., 1984) and LGL (Herman and Rabson, 1984). In fact, this mechanism of LPS-stimulated IL-2 production has been recently demonstrated in human peripheral blood mononuclear cells (Le et al., 1986).

In addition to the indirect effect of LPS on the enhancement of NK cytotoxicity, LPS may also exert a direct effect on human NK cells as indicated by the increased NK cytotoxicity of sorted Leu-11a$^+$ cells and the incorporation of LPS by these cells (Kang et al., 1988).

Results from our studies also showed that IL-2 significantly enhanced NK cytotoxicity against K562 targets and caused hypertrophy of Golgi complex and rough endoplasmic reticulum, and mitosis in addition to the increases in electron-dense granules in Leu-11a$^+$ cells. Similar observations of the effect of IL-2 on the ultrastructure of NK cells have also been recently reported in Percoll purified human LGL (Zarcone et al., 1987). All these ultrastructural changes are believed to be implicated in the enhancement of NK cytotoxicity. In addition, the observation of TRI in NK cells following treatment with IL-2 confirms that IL-2 stimulates production of interferon in human PBMC (Handa et al., 1983).

Although both IFN-r and IL-2 have been reported to enhance NK cytotoxicity, the ability of IL-2 to directly affect the cytolytic activity of NK cells has been controversial. Ortaldo et al. (1984) and others (Shiiba et al., 1984; Weigent et al., 1983) reported that the enhancement of cytolytic activity in NK cells by IL-2 is a consequence of triggering IFN-r production. On the other hand, Trinchieri et al. (1984) and other investigators (Svedersky et al., 1984; Van de Griend et al., 1986; Kabelitz et al., 1985) suggested that IL-2 induced enhancement of NK cytolytic activity is IFN independent since antibodies against IFN-r do not prevent enhancement of cytolytic activity by IL-2.

In summary, results from the present studies indicated that: (1) both LPS and IL-2 effectively enhance NK cytotoxicity in PBMC against K562 tumor cells; (2) both LPS and IL-2 cause similar ultrastructural changes in Leu-11a$^+$ NK cells; these changes correlate with NK activity; (3) the effect of LPS on the enhancement of NK cytotoxicity may be a direct and/or indirect process; (4) interferon is implicated in the augmentation of cytotoxicity by LPS; and (5) IL-2 stimulates mitosis of both Leu-7$^+$ cells and Leu-11a$^+$ cells.

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Discussion with Reviewers

P. Grimley: How do the authors explain the striking difference in size of the carboxylate beads as observed by different methods (Figs. 2A, 2B)? It is not entirely clear from the text whether all cells are identically fixed both for SEM and TEM work, or whether the critical point drying method may have been employed?

Authors: Micrographs of Figs. 2A and 2B showing binding of carboxylate beads to the surface of Leu-11a$^+$ cells were not prepared at the same magnification and enlargement. The magnification of Fig. 2B is slightly higher than that of Fig. 2A. Samples for SEM and TEM were fixed identically with 1% glutaraldehyde/1% paraformaldehyde. Cells for SEM were critical point dried using liquid CO_2.

P. Grimley: Is there any evidence that LPS induces interferon directly in the sorted Leu-11a$^+$ cells or is a mixture of PBMC subtype required? Have there been any tests to determine the specific type or relative proportions of interferons induced by LPS in these or other experiments?
Authors: We have no direct evidence to show that sorted Leu-11a$^+$ cells produced interferons after stimulation with LPS. We did not assay the supernatant from the culture of sorted Leu-11a$^+$ cells with LPS. However, reports have indicated that Leu-11a$^+$ cells may produce interferons following stimulation with LPS. Studies show that interferons are produced by LGL in which 90% of the cells express Leu-11a phenotype as stated in the Discussion section. Interferons can also be produced by T cells and monocytes/macrophages stimulated with LPS. Gamma interferon has been reported to be produced by T cells and NK cells stimulated with LPS (Blanchard et al., 1986; Matsumura and Nakano, 1988).

C. Bucana: What proportion of Leu-11a$^+$ cells are phagocytic in (a) freshly isolated preparations and (b) in the IL-2 and LPS treated preparations?
Authors: Approximately 15% of Leu-11a$^+$ cells from freshly prepared PBMC displayed ingestion of bacteria as compared to 36% of Leu-11a$^+$ cells from LPS-treated PBMC (Kang et al., 1988). We did not perform the study on the effect of IL-2 on the phagocytic activity of Leu-11a$^+$ cells.

C. Bucana: With reference to the granule formation in IL-2 treated cells, did you observe degranulation when these cells reacted with tumor target cells or when these cells phagocytosed bacteria?
Authors: We did not particularly focus on the degranulation in the present study. Degranulation has been reported in NK cells when NK cells are in contact with target cells (Neighbour et al., 1982). Although there was no statistical information on the numbers of granules in Leu-11a$^+$ cells that phagocytosed bacteria, we did observe fewer or no dense granules in these cells.

C. Bucana: Was thymidine incorporation done on cells treated with IL-2 or LPS? It is difficult to conclude that IL-2 stimulates proliferation of NK cells without an actual increase in cell numbers.
Authors: Tritiated thymidine incorporation by Leu-11a$^+$ cells was only performed in PBMC treated with LPS for 24 hours. Results showed that no incorporation of thymidine was observed in Leu-11a$^+$ cells (Kang et al., 1988). In fact, numerous studies have indicated that IL-2 stimulates proliferation of NK cells (Phillips and Lanier, 1986; Trinchieri et al., 1984; Timonen et al., 1982a; London et al., 1986). We did observe that 18% of the total peripheral blood lymphocytes were Leu-19$^+$ cells after 4 days incubation with IL-2 as compared to 3% Leu-19$^+$ cells in the PBMC without treatment with IL-2

C. Bucana: In Fig. 4, how do you determine the degree of exposure needed to demonstrate gold particles in double labeled cells? Was this verified by X-ray analysis?
Authors: Colloidal gold has high electron density and is clearly visible with HRP reaction on the cell surface of labeled cells in the negatives. However, the normal exposure time for a continuous tone micrograph always obscures the appearance of gold grains. Therefore, an underexposure procedure is required to demonstrate gold particles on the cell surface. In this case, a normally exposed continuous tone micrograph was initially prepared to determine the reduction of exposure time. The normal exposure time was gradually decreased until individual gold grains were clearly shown in the peripheral surface of the cells. X-ray microanalysis was not performed.

H. Gamliel: There is only limited information on the rationale of testing whole PBMC vs Leu-11$^+$. Is it not better to compare Leu-11$^-$ instead?
Authors: It is well documented that cells expressing Leu-11 antigen are the most potent NK cells in human peripheral blood (Lanier et al., 1983; Phillips and Babcock, 1983). The aim of the present study is to elucidate the possible mechanism of how LPS and IL-2 stimulate NK activity. This is why Leu-11$^+$ cells were chosen for the present study. It is known that LPS stimulates production of interferons by PBMC and interferons stimulate NK cell mediated cytotoxicity as stated in the Discussion of the paper. The reason why we treated sorted Leu-11$^+$ cells with LPS was to elucidate whether LPS has a direct effect on purified Leu-11$^+$ cells with respect to NK activity. Results showed that LPS does have a direct stimulating effect on the NK activity of Leu-11$^+$ cells in addition to morphological effects. This is also confirmed by incorporation of LPS by Leu-11$^+$ cells (Kang et al., 1988).

H. Gamliel: From the Abstract, and Materials and Methods it seems that Leu-11$^+$ cells were not treated with IL-2, while the results bring data on Leu-11$^+$ cells treated with IL-2. Do the results on Leu-11$^+$ (after IL-2) refer only to Leu-11$^+$ cells from the IL-2 treated PBMC? If yes, why did you not treat isolated Leu-11$^+$ cells with IL-2? or at least why was this possibility not reviewed here?
Authors: It is correct that we did not treat sorted Leu-11$^+$ cells with IL-2. In this report, the IL-2 stimulated Leu-11$^+$ cells were, in fact, from IL-2 treated PBMC. The major aim of the present study is to correlate IL-2 induced morphological changes in Leu-11$^+$ cells with functional alterations following IL-2 stimulation in an attempt to elucidate the mechanism by which IL-2 enhances NK activity. Leu-11$^+$ cells were chosen for ultrastructural study because these cells are the most potent NK cells. The effect of IL-2 on Leu-11$^+$ cells and Leu-11$^-$ cells has been recently reported in adult peripheral blood and cord blood. Seki and his coworkers (1985) reported that IL-2 significantly augmented NK cytotoxicity of Leu-11$^+$ cells, but not Leu-11$^-$ cells.

H. Gamliel: Several studies have shown that Leu-11$^+$ are precursors of lymphokine activated killer (LAK) cells, and when incubated with IL-2, they become potent LAK cells that induce lysis of tumor targets. Do the results of this study refer to the same cell

population (in IL-2 treated PBMC), and how do the functional and ultrastructural changes seen after LPS treatment of Leu-11$^+$ relate to the LAK definition of cells?

Authors: Itoh et al. (1985) have shown that precursors of recombinant IL- 2 activated killer cells which are cytotoxic for solid tumor cells are a subset of NK cells with Leu-7$^-$/Leu-11$^+$ membrane phenotype. They have shown that IL-2 stimulated sorted Leu-11$^+$ cells displayed significant increases in cytotoxicity against both noncultured melanoma tumor cells and K562 tumor cells. However, no significant increases in the cytotoxicity against noncultured melanoma tumor cells were found in non-IL-2 stimulated sorted Leu-11$^+$ cells which had high NK activity against K562 tumor cells. This clearly indicates that IL-2 stimulates production of NK cells capable of lysing target cells which are not susceptible to non-stimulated Leu-11$^+$ cells. In our studies, we did observe that Leu-11$^+$ cells from IL-2 stimulated PBMC displayed significant increases in cytotoxicity against K562 cells as compared to Leu-11$^-$ cells from the same PBMC sample (data not included in this paper). Based on our observations and other reports, the IL-2 activated Leu-11$^+$ cells are possibly overlapping LAK cells. In the present study, PBMC were only treated with LPS for 24 h. Whether sufficient amounts of IL-2 were produced by T cells via stimulation of IL-1 produced by monocytes is not known. The augmentation of cytotoxicity by LPS in LPS-treated PBMC is possibly attributed to production of interferons in the PBMC instead of interleukins.

H. Gamliel: Can you elaborate on the extent and/or importance of specific receptors for LPS or IL-2 for the ultrastructure/functional changes to be induced?

Authors: It is well established that many cellular activities are triggered by the interaction of ligand with receptors. For example, LPS stimulates monocytes to produce some important mediators related to the pathogenesis of septic shock. No reports have shown receptors for LPS in human NK cells. We previously observed binding of LPS to Leu-11a$^+$ cells and ingestion of LPS by these cells (Kang et al., 1988). The binding of LPS to the cell surface of Leu-11a$^+$ indicates the possible existence of membrane receptors although receptor study has not been done in the present report. A recent report indicates that LPS and gamma interferon strongly increase IL-2 receptors on human peripheral monocytes (Wahl et al., 1987). Specific IL-2 receptors have been reported in T cells and NK cells (Cantrell and Smith, 1984). Internalization of IL-2 has been shown in thymoma cells following binding to its receptors. The interaction of IL-2 with its receptors elicits enhancement of NK cytotoxicity in short-term culture (Lanier et al., 1985) and proliferation of lymphokine-activated killer cells to lyse cultured solid tumor cells usually insensitive to classical NK cell killing (Trinchieri et al., 1984). Anti-Tac monoclonal antibody which recognizes IL-2 receptors, does not block the recombinant IL-2 induced increase of NK activity, but blocks the proliferation of NK cells once activated (Ortaldo et al., 1984). This result suggests the possible existence of two different receptors or binding sites. Indeed, reports have shown IL-2 receptors consist of at least two different molecules, one being the 55 kda Tac-binding molecule and the other a 75 kda IL-2 binding molecule, the high affinity portion of the IL-2 receptor complex (Tsudo et al., 1986). With respect to the effect of IL-2 receptor on cell proliferation, NK cells and T cells respond differently to IL-2. NK cells respond directly to IL-2 by strong proliferation. Some T cells require the presence of IL-1 to display proliferative response to IL-2 (Aribia et al., 1987) since IL-1 promotes the expression of functional Tac$^+$ IL-2 receptors on these cells (Lowenthal et al., 1986).

C. Grossi: This reviewer is only concerned about one point which is reiterated throughout the manuscript (Abstract, Discussion), i.e., that IL-2 induces proliferation of all LGL subsets. The only evidence for this concept is provided in Fig. 13B showing a mitotic figure. However, there is ample evidence in the literature to support the contention that IL-2 induced enhancement of cytotoxicity is not related to cell proliferation. The Reviewer would like to see a quantitation (e.g., by tritiated thymidine uptake) of the direct proliferative effect of IL-2 on otherwise unstimulated Leu-7$^+$, Leu-11$^+$ and Leu-19$^+$ cells.

Authors: As indicated by Dr. Grossi, we did observe mitotic figures in Leu-7$^+$, Leu-11$^+$, and Leu-19$^+$ NK subsets 4 days after stimulation with IL-2. The numbers of each subset from the IL-2 treated PBMC for 4 days were increased as compared to those of the non-IL-2 treated samples. For instance, 18% of the total lymphocytes (monocytes were excluded) displayed Leu-19 antigen as compared to 3% in the non-IL-2 treated control. Although thymidine incorporation study was not conducted in the present report, the results clearly indicated that IL-2 stimulated proliferation of NK subsets. In addition, many reports have shown that IL-2 directly activates proliferation of LGL (Phillips and Lanier, 1986; London et al., 1986). We totally agree with Dr. Grossi that IL-2 induced enhancement of NK activity is not related to cell proliferation. Our unpublished data did show that 89% specific lysis were observed two days after IL-2 stimulation compared to 54% specific lysis in unstimulated samples. In the same samples, 12.7% of the total lymphocytes were found to be Leu-11$^+$ cells in the unstimulated samples, while only 9.7% of lymphocytes expressed Leu-11 antigen 2 days after IL-treatment. Study on the incorporation of tritiated thymidine by NK cells of different subsets following IL-2 stimulation will be initiated in the near future.